SELCTION AND EVALUATION OF CANDIDATE BACILLUS-BASED DIRECT-FED MICROBIALS FOR USE IN COMMERCIAL POULTRY PRODUCTION

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ABSTRACT

Increasing pressure from consumers and government regulatory agencies has led an ever increasing number of U.S. poultry producers to reduce or eliminate the use of antibiotics in their operations. This has directly resulted in reduced overall performance and an increase in flock health issues. One possible alternative may be an effective *Bacillus*-based direct-fed microbial that reduces enteric health issues and improves overall production parameters. To select for potential direct-fed microbial isolates belonging to the genus *Bacillus*, environmental samples were pasteurized, plated, and evaluated for anti-microbial activity using soft agar overlays containing target bacterial pathogens. Colonies which produced anti-*Salmonella* activity were isolated and then evaluated for *in vitro* anti-clostridial and anti-*Campylobacter* activity using similar soft agar overlays under appropriate atmospheres. Polyvalent isolates were speciated and both nonpathogenic and/or GRAS species were further evaluated for resistance to high temperatures and for the ability to grow to high numbers with high sporulation efficiency (10^{10} spores per gram or greater) in a solid state media. Isolates PHL-MM65 and PHL-NP122 (a *Bacillus laterosporus* and *Bacillus subtilis* respectively) were further evaluated using poults raised under commercial conditions. After 7d of conventional brooding, 480 poults from within the house were tagged, weighted, and placed into one of four replicate pens for each treatment group (negative control, nitarsone (an organic arsenical), PHL-MM65 10^6 spores/g feed, or PHL-NP122 10^6 spores/g feed). After 23 days the poults were weighed and body weight was calculated for each group, PHL-NP122 (853g), and histostat (852 g) were found to be heavier ($p \leq .05$) than the negative control (784g), while PHL-MM65 (794g) was not significantly heavier. Also at day 23 of the trial, the ceca were aseptically removed from 10 euthanized poults per pen and cultured for recovery of *Salmonella*. Treatment with *Bacillus* isolates PHL-NP122 and PHL-MM65 resulted in a significant reduction ($p \leq .05$) in the percentage of poults colonized by *Salmonella* (17.5% and 23.3% respectively) as compared to the negative control (47.5%). These data may suggest that this method of screening and evaluation could lead to commercially useful *Bacillus*-based probiotics.

INTRODUCTION

Non-typhoid *Salmonella* infections of humans result in an estimated 1.4 million cases of salmonellosis, resulting in approximately 600 deaths in the United States each year (1,2). Raw or undercooked poultry products, eggs, and meat, have often been implicated in cases of salmonellosis (3, 4). One way to reduce the incidence of *Salmonella* contaminated poultry products, and therefore the incidence of salmonellosis in people, may be through the use of probiotics which can effectively reduce the level of *Salmonella* within the alimentary tract of live poultry.

Probiotics, frequently involving lactic acid bacteria, have been in use since the turn of the twentieth century and the benefits of these cultures in terms of pathogen reduction and increased gastrointestinal health have been well documented. The widespread use of these products in animal health applications has been limited, largely due to issues with stability, application, and cost. The development of a low cost, stable, efficacious probiotic that can be administered in animal feed is needed to increase the use of these products.

Bacteria of the genus *Bacillus* are gram positive, endospore forming, aerobic (or facultative anaerobic), and rod-shaped. Currently there are approximately 80 species of *Bacillus*, however the classical *Bacillus* genus has more recently been separated into 25 genera consisting of over 200 species (5). *Bacillus* species have long been used to produce aerobically fermented foods such as natto (a fermented soy bean product) and soumbala (a fermented locust bean product) in Asia and Africa respectively. More recently this genus has been used in probiotic formulations for both human and veterinary applications to treat gastrointestinal ailments. Some of these products have been shown to kill enteropathogenic bacteria such as *Salmonella*, *Escherichia coli*, and *Clostridium perfringens in vitro* as well as to reduce the number of these organisms *in vivo*. Additionally, some *Bacillus* probiotics have been shown to increase growth rate and improve feed efficiency in poultry and swine (6,7,8).

The main advantage *Bacillus*-based probiotics have over the more traditional lactic acid bacteria probiotics stems from the stability of the endospore formed by *Bacillus* and closely related genera. The endospore is a survival form of the organism and is resistant to desiccation, UV light, many chemical disinfectants, high osmotic pressures, salinity, acidity, and heat. In addition, *Bacillus* endospores have been shown to be viable for decades in the laboratory, and some authors have reportedly isolated viable endospores from mineral deposits formed millions of years in the past. One recent study indicated successful isolation of a *Bacillus* from a 250 million year-old salt crystal (9). This stability enables these probiotic products to be stored at room temperature for long periods of time without loss of viability. Additionally, this stability enables the endospore to go through the feed milling process with only minimal loss of viability. New methods in solid-state fermentation allow for the production of large numbers of endospores using relatively inexpensive processes (10). The combination of new fermentation methods for reduced cost of production, combined with extreme stability in feed, may provide improved opportunities for the use of effective *Bacillus* spores which provide pathogen reduction and improved performance.

MATERIALS AND METHODS

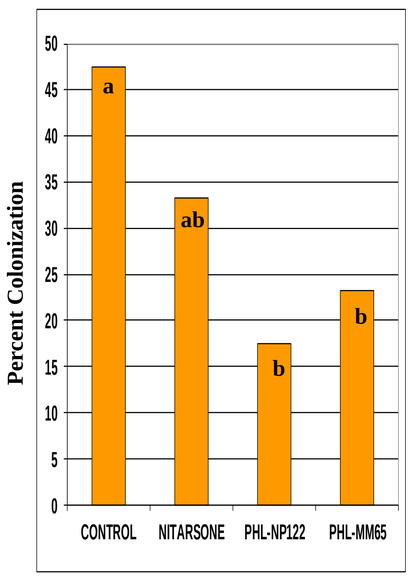
Isolation and Selection of *Bacillus* Isolates:
Environmental samples were obtained for the purpose of screening for *Bacillus* spores. Samples were diluted with sterile saline, then pasteurized by heating at 70°C for 10 minutes in 15 mL conical plastic tubes. Samples were then plated onto a solid agar medium developed in our laboratory which provides nitrogen solely in the form of ammonium sulfate. *Bacillus* strains identified using these methods were then purified to obtain a monoculture and then specifically identified using the BioMerieux API 50 CHB *Bacillus* identification system.
Isolates were grown on a modified version of the solid state fermentation medium developed by Zhao et al (10). Briefly, wheat bran and rice hulls were combined and the ammonia medium described above, without agarose, was added to the mixture to achieve a moisture content of 40% by weight. Isolates were added to the medium, and grown at 37°C for 24 h and then allowed to incubate for an additional 72 hours at 30°C. The cultures were dried at 60°C and then ground. Samples were heat shocked by incubation at 70°C for 10 minutes to kill any remaining vegetative cells and spores were enumerated by serial dilution and colony counting.

Evaluation of *Bacillus* Isolates as Potential Probiotics
In commercial turkey housing, turkey poults were reared conventionally for 6 days. On the seventh day prior to the removal of brooder rings, a sample of 100 turkey poults were weighed. 480 poults, within 1 standard deviation of the mean of the sample group, were assigned to a group then tagged. Sixteen 1.22 x 1.22 m wire panel pens were constructed in 4 blocks of 4 pens down the center of the house. The groups were block randomized and 30 tagged poults were randomly placed into each pen. Each pen was equipped with a single manual feeder and an automatic waterer. Poults were fed a commercial turkey starter diet with no supplementation, 0.01875% nitarsone, or candidate *Bacillus* DFM PHL-NP122 or PHL-MM65 at the rate of 10^6 spores/g finished feed. Both nitarsone and the DFM candidates were fed continuously throughout the trial and control group poults received no treatment. Poults were weighed individually on days 1, 14, and 23 of the trial. Mean body weights (BW) and body weight gain (BWG) were used to evaluate DFM efficacy on production parameters. On day 23, 10 poults per pen were humanely killed by cervical dislocation at the farm. The ceca were aseptically removed and transported on ice to the Poultry Health Laboratory at the University of Arkansas. The cecal samples were macerated and diluted 1:4 with sterile saline. The samples were enumerated using 10-fold dilutions and plate-counting following overnight incubation at 37°C on brilliant green agar (BGA) with 25 µg/mL novobiocin, MacConkey agar, and Rogosa SL Agar for enumeration of *Salmonella*, coliforms, and oxygen tolerant lactic acid bacteria respectively. Tetrathionate broth was added to the cecal samples and incubated for 24h for enrichment of *Salmonella*. The enriched samples were struck onto BGA with 25 µg/mL novobiocin and incubated overnight. All plates were evaluated for colonies characteristic of *Salmonella*, coliforms, or lactic acid bacteria on the appropriate selective media.

RESULTS

Two isolates, PHL-NP122 (*Bacillus subtilis)* and PHL-MM65 (*Bacillus laterosporus),* were able to use ammonia as their sole source of a nitrogen and were also able to produce spores to a high number (greater that 10^{11} spores per gram). These two isolates were selected for further evaluation as potential probiotics in a field trial after several *in vivo* laboratory trials (data not shown). In this experiment, body weight, *Salmonella* infection, enumeration of coliform bacteria, and enumeration of lactic acid bacteria were evaluated. Spores of these isolates were added to a standard commercial turkey starter ration at the following rate of 10^6 cfu per gram of feed. There was a significant ($p \leq 0.05$) reduction in percent *Salmonella* positive cecal tonsils at 23 days of age from birds treated with *Bacillus* PHL-NP122 (17.5%) and PHL-MM65 (23.3%) as compared to non-treated controls (47.5%). Additionally, there was also a significant reduction in number *Salmonella* recovered in the poults treated with PHL-NP122 (0.47 log10 cfu/g) as compared to the negative control (1.92 log10 cfu/g). There was not a significant difference in rate of colonization with PHL-MM65 (0.89 log10 cfu/g). Neither candidate probiotic had a significant impact ($p \geq 0.05$) on the numbers of coliforms or lactic acid bacteria in the ceca. Treatment with isolate PHL-NP122 also had a significant ($p \leq 0.05$) positive impact on body weight (852.8 g) as compared to the control group (784.2) and isolate PHL-MM65 (793.9 g). The BW and BWG of the PHL-NP122 treated group were not significantly ($p \leq 0.05$) different than the group treated with the organic arsenical nitarsone.

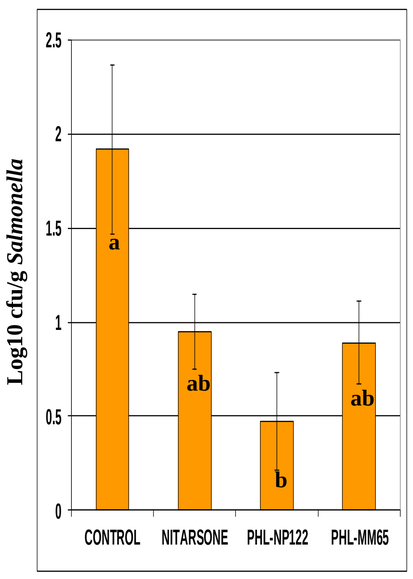
SALMONELLA CECAL TONSIL COLONIZATION



Treatments

Seven day old turkey poults were assigned to one of four dietary treatments (4 pens each) down the center of a commercial turkey house. Treatments included: unmedicated base ration, 0.01875% nitarsone, or one of two candidate *Bacillus* probiotics isolates (PHL-NP122 or PHL-MM65) at the rate of 10^6 spores per gram of feed. On day 23 of the experiment the cecal tonsils of 10 poults per pen of 30 were cultured for isolation of *Salmonella.* Different letters denote significant differences ($p \leq 0.05$).

LOG10 CFU *SALMONELLA PER GRAM* CECAL CONTENT



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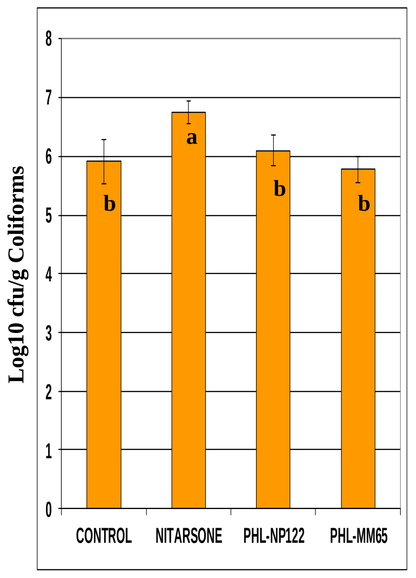
LOG10 CFU *COLIFORMS PER GRAM* CECAL CONTENT



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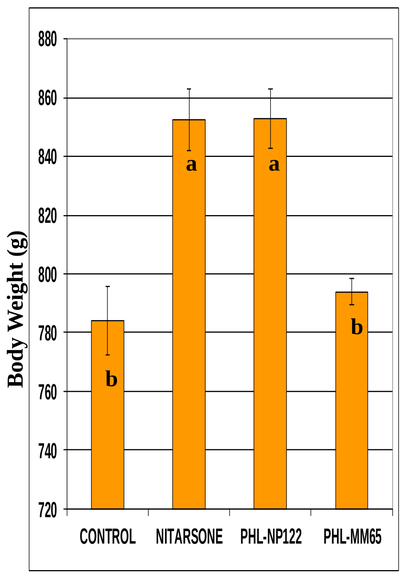
BODY WEIGHT DAY 23



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CONCLUSIONS:

The *Bacillus subtilis* isolate PHL-NP122 reduced both the incidence and amount of *Salmonella* isolated from the cecal tonsils in this trial, and both body weight and bodyweight gain were increased by this same isolate. Neither isolate, PHL-NP122 nor PHL-MM65, significantly altered the number of lactic acid bacteria or coliforms present in the ceca. Additionally, the ability of this isolate to produce spores in excess of 10^{11} spores per gram, on a relatively cheap medium, as well as the ability of these spores to withstand temperatures higher than those traditionally used in the feed milling process, suggests that isolate PHL-NP122 should be further studied as a candidate for use in the poultry industry. Continued screening is currently ongoing in our laboratories for other *Bacillus* isolates with greater efficacy for reduction of enteric *Salmonella* infections as well as isolates that can reduce enteric clostridial loads.

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